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# Hepatocyte swelling leads to rapid decrease of the G-/total actin ratio and increases actin mRNA levels

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Exposure of isolated rat hepatocytes to hypotonic (190 mosmol/l) incubation media lowered the cellular G-actin level without affecting the total actin content: here the G-/total actin ratio decreased by 15.5 ± 1.4% ($n$=7). Similar effects were observed following isotonic cell swelling by either addition of glutamine (10 mM) or insulin (100 nM), resulting in a decrease of the G-/total actin ratios by 13.5 ± 2.1% ($n$ = 5) and 14.1 ± 1.1% ($n$ = 11), respectively. The effects of hypotonic exposure, glutamine and insulin on the G-/total actin ratio largely occurred within 1 min and persisted for at least 2 h in presence of the respective effectors. After a 120 min exposure to hypotonic media, glutamine or insulin the actin mRNA levels were increased 2.4-, 2.0- and 3.6-fold, respectively. Hypertonic exposure lowered the G-/total actin ratio by only 4.9 ± 2.5% ($n$ = 4) and increased actin mRNA levels only 1.2-fold. There was a close relationship between glutamine- and hypotonicity-induced cell swelling and the decrease of G-/total actin ratios. The data suggest that cell swelling exerts rapid and marked effects on the state of actin polymerization and increases actin mRNA levels. Thus, cytoskeletal alterations in response to cell swelling may be involved in the regulation of hepatic metabolism by cell volume.

Cell swelling; Glutamine; Cell volume; Isolated hepatocyte; Insulin; Anisotonicity; Hypotonic; Hypertonic; Actin; Gene expression; Cytoskeleton

## 1. INTRODUCTION

Cell volume changes were recently recognized as potent modulators of metabolic liver cell function and evidence has been presented that cell volume changes, as they occur in response to hormones and amino acids act like a 'second or third messenger' modifying hepatic metabolism (for review see [1]). Although the mechanisms linking cell volume changes to cellular function are poorly understood, cytoskeletal alterations may play a role. Indeed, the polymerization state of microfilaments, which can change under the influence of growth factors and other hormones by shifting the actin monomer (G)/polymer (F) actin equilibrium, was repeatedly suggested to be important for cellular function [2–8]. On the other hand, volume-regulatory responses in some cell types are blunted by inhibitors of the microfilament system [9,10] and there is some microscopic evidence on a reorganization of microfilaments following hypotonic cell exposure [10,11]. Further, hypotonic exposure was shown to increase mRNA levels of actin in rat liver [12].

Accordingly, we addressed the question whether cell volume changes exert effects on the polymerization state of actin and whether such effects also occur during isotonic cell swelling. The data show that cell swelling leads to a rapid decrease of the G/total actin ratio, suggesting rapid polymerization of G-actin and to an increase of actin mRNA levels.

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## 2. MATERIALS AND METHODS

### 2.1. *Isolation and incubation of isolated rat hepatocytes*

Isolated rat hepatocytes were prepared according to [13] from livers of male Wistar rats, fed ad libitum on stock diet (Altromin), following collagenase perfusion. After washing, the hepatocytes were incubated at 37°C in a Krebs-Henseleit buffered saline (final volume 4 ml) supplemented with 6 mM glucose. The incubations were continuously gassed with water-vapor saturated $O_2/CO_2$ (95:5 v/v). Viability of hepatocytes as assessed by Trypan blue exclusion at the end of the 2 h-incubations was about 90%. The protein content in the incubations was 3–6 mg/ml. The incubation volume was 4 ml. Hepatocytes were preincubated for 20 min with the above-mentioned medium; then ($t$ = 0 min), glutamine, hormones or anisotonic conditions were instituted. Samples for determination of G- and total actin and protein were taken either after 1, 20 or 120 min. Glutamine and insulin were added to the incubations to give concentrations of 10 mM or 100 nM, respectively. In order to maintain maximally active hormone concentrations throughout the 2 h-incubation period, insulin additions were repeated every 30 min. In control incubations the osmolarity was 310 mosmol/l, anisotonic conditions were achieved by adding or removing appropriate amounts of NaCl, if not indicated otherwise.

### 2.2. *DNase I inhibition assay for actin determinations*

Actin in monomeric and filamentous form was measured in the cells using the G-actin-dependent DNase I inhibition assay as previously described in [14], with the following modifications: $10^6$ cells, washed twice with phosphate-buffered saline (PBS), pH 7.4, were treated with 100 μl lysis buffer containing 10 mM $K_2HPO_4$, 100 mM NaF, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM dithiothreitol, 0.5% Triton

X-100, 1 M sucrose, pH 7.0. For determination of the G-actin content 10 μl of the lysate were added to the assay mixture containing 7 μl DNase I solution (1 mg/10 ml DNase I in 50 mM Tris-HCl, 10 mM phenylmethyl-sulfonylfluoride (PMSF), 0.5 mM $CaCl_2$, pH 7.5) and 1 ml DNA solution (4 mg/100 ml DNA in 100 mM Tris-HCl, 4 mM $MgSO_4$, 1.8 mM $CaCl_2$, pH 7.5). The DNase I activity was monitored continuously in a quartz cuvette with a Sigma-ZWS II double beam spectrophotometer at 260 nm. Actin in the sample was quantitated by reference to a standard curve for the inhibition of DNase I activity, prepared using rabbit muscle G-actin, isolated as previously described [15]. Actin concentration was determined by the optical density at 290 nm using the extinction coefficient $\varepsilon_{290} = 26460\ mol^{-1}\ cm^{-1}$. A linear relationship was observed over the range of 26–68% inhibition of DNase I activity. To measure total actin, aliquots of the lysed cell suspension were diluted 2–3 times with lysis buffer and then incubated for 20 min with an equal volume of guanidine-HCl buffer (1.5 mM guanidine-HCl, 1 M sodium acetate, 1 mM $CaCl_2$, 1 mM ATP, 20 mM Tris-HCl, pH 7.5) to depolymerize F-actin to monomeric G-actin. The G-actin content in the hepatocyte suspensions was related to the total protein content. Protein was determined with the biuret reaction described in [16].

### 2.3. *Northern blot analysis of actin mRNA*

The isolation and size-fractionation of total cellular RNA was performed exactly as previously described [17]. After transfer of RNA to Gene Screen nylon membranes (New England Nuclears, Boston, USA) the filters were prehybridized and hybridized with cDNA probes $\gamma$-$^{32}$P-labeled by random priming, using oligodeoxynucleotides (specific activity $5\times10^7$ cpm/μg), according to [18]. The β-actin mRNA levels were normalized to the level of glutamate dehydrogenase mRNA. Hybridizations were carried out as described [19]. Washed filters were exposed to Kodak XR film at −80°C in the presence of an intensifying screen. Hybridization was quantified by a computed Image Analyzer (Quant 970, Cambridge Instruments, Cambridge, UK). The probes used were a rat β-actin cDNA *Pst*I fragment inserted into a pUC 18 plasmid [20] and a human glutamate dehydrogenase cDNA probe [21], kindly provided by Dr. G. Mavrothalassitis (Research Center of Crete).

### 2.4. *Determination of hepatocyte volume*

The intracellular water space of isolated rat hepatocytes was determined as described previously [22] using the method by [23] with some modifications: i.e. [$^3$H]inulin was used as extracellular marker and [$^{14}$C]urea as marker for intra- plus extracellular space as previously done and validated in studies on the intact liver [24]. In brief, hepatocytes were incubated in the above-mentioned medium for 120 min and then [$^3$H]inulin (20 kBq) and [$^{14}$C]urea (20 kBq) were added to the incubations. 5 min later a 2.5 ml-aliquot of the cell suspension was gently centrifuged (170×g) for 30 s. $^3$H and $^{14}$C radioactivity was determined by scintillation spectrometry in the supernatant and the pellet, respectively. The intracellular water space was calculated from the $^{14}$C radioactivity found in the pellet after correction for residual extracellular water in the pellet by using the specific radioactivities in the supernatant. In the individual cell preparation the intracellular water space ('cell volume') found in control incubations was set to 100% and the volume-effects of anisotonicity, glutamine and insulin are expressed as percentage hereof. In the absence of further additions ('control'), cell volume was 2.08 ± 0.02 μl/mg protein ($n$ = 13) after a 120 min incubation.

### 2.5. *Expression of results*

If not indicated otherwise, the G-/total actin ratio in control incubations obtained from a cell preparation was set to 100% and the influence of effectors on actin polymerization is given as percentage hereof. In addition data from different cell preparations are given as G-actin content per mg total hepatocyte protein. Respective data obtained from different cell preparations were statistically treated by ANOVA and unpaired $t$-test; they are given as means ± SEM ($n$ = number of cell preparations) and $P<0.05$ were considered statistically significant.

### 2.6. *Materials*

[$^3$H]Inulin and [$^{14}$C]urea were from Amersham Buchler (Frankfurt, Germany). Collagenase was from Biochrom (Berlin, Germany). Insulin (from bovine pancreas), DNA (from herring testes) and DNase I (from bovine pancreas; 600 kU/mg protein) were from Sigma (Munich, Germany). All other chemicals were from usual commercial sources in the purest grade available.

## 3. RESULTS AND DISCUSSION

### 3.1. *Effect of hypotonic exposure, glutamine and insulin on actin polymerization*

In normotonic control incubations, the G-actin content was 21.2 ± 0.7 μg/mg total hepatocyte protein ($n$ = 22), in close agreement with earlier published data by others [4]. The ratio of monomeric (G-)actin to total cellular actin; i.e. the G-/total actin ratio was 0.250 ± 0.006 ($n$ = 22) and did not significantly change over a 120 min incubation period (for data see legend Table I). Upon hypotonic incubation, due to lowering the NaCl concentration, the G-/total actin ratio significantly decreased (Table I), suggesting a stimulation of actin polymerization following hypotonic exposure. In line with this interpretation, the total actin content did not significantly change upon hypotonic exposure (Table II). As further shown in Table I, this response was already observed after 1 min of hypotonic exposure and persisted up to 2 h of hypotonic exposure. Here, it should be noted that volume-regulatory net $K^+$ efflux from the hepatocytes is terminated within the first 8 min

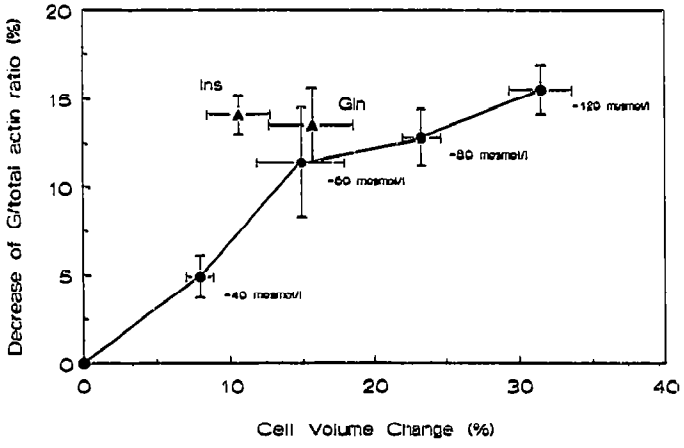


Fig. 1. Relationship between glutamine-, insulin- and hypotonicity-induced cell swelling and the accompanying decrease of G/total actin ratio. Data on cell volume and G-/total actin ratio were obtained from cells being exposed for 120 min to hypotonic fluid (190, 230, 250, 270 mosmol/l), glutamine (Gln; 10 mmol/l) or insulin (Ins; 100 nmol/l). The curve drawn in the figure takes into account only data obtained following hypotonic exposure. Data are given as means ± SEM and are from 3–10 different cell preparations.

Table I

Effect of anisotonic exposure, glutamine, insulin and glucagon on G-/total actin ratio in isolated rat hepatocytes

Hepatocytes were preincubated as described in the methods section for 15 min. Then ($t$ = 0 min) anisotonicity, glutamine, insulin or glucagon was instituted and cells were assayed for G- and total actin after $t$ = 1, 20 and 120 min. Anisotonic conditions were achieved by lowering or increasing the NaCl concentration. In normotonic control incubations the osmolarity was 310 mosmol/l. In normotonic control incubations the G-/total actin ratio did not change significantly during the 120 min incubation period and was 0.245 ± 0.026 ($n$ = 3), 0.244 ± 0.011 ($n$ = 4) and 0.250 ± 0.006 ($n$ = 22) after 1, 20 and 120 min of incubation, respectively. Data reflect the percent change of the G-/total actin ratio induced by the effectors and are given as means ± SEM ($n$=number of cell preparations).

| Duration of exposure | 1 min | 20 min | 120 min |
|---|---|---|---|
| | % change of G-/total actin ratio | | |
| Hypotonic (270 mosmol/l) | – | – | − 4.9 ± 1.2 (4) |
| (250 mosmol/l) | – | – | −11.4 ± 3.1 (4) |
| (230 mosmol/l) | – | – | −12.6 ± 1.6 (4) |
| (190 mosmol/l) | −10.8 ± 2.3 (3) | −18.4 ± 2.7 (4) | −15.5 ± 1.4 (7) |
| Hypertonic (430 mosmol/l) | + 0.3 ± 2.3 (3) | − 2.5 ± 5.3 (3) | − 4.9 ± 2.5 (4) |
| Glutamine (10 mmol/l) | −12.9 ± 4.1 (3) | −11.9 ± 1.4 (3) | −13.5 ± 2.1 (5) |
| Insulin (100 nmol/l) | −10.3 ± 7.0 (3) | −12.0 ± 7.1 (4) | −14.1 ± 1.1 (11) |
| Glucagon (100 nmol/l) | + 1.7 ± 5.7 (3) | − 4.1 ± 3.5 (3) | − 7.1 ± 3.8 (3) |

of hypotonic exposure, however, this volume-regulatory response does not restore the initial cell volume completely, instead the liver cells remain in a swollen state [25]. The extent of stimulation of actin polymerization was dependent upon the extent of osmolarity decrease in the perfusion medium (Table I). Hypertonic exposure, on the other hand had, if at all, only a slight effect on the G/total actin ratio.

Glutamine was recently shown to induce cell swelling within 1–2min due to a cumulative uptake of the amino acid into the cells driven by the electrochemical sodium gradient across the plasma membrane [26]. Cell swelling is also induced by insulin due to cellular accumulation of $K^+$, $Na^+$ and $Cl^-$ [27,28]. As shown in Table I, both glutamine and insulin rapidly decreased the G/total actin ratio. The decrease of the G/total actin ratio under the influence of insulin as determined by the decrease of G-actin content is quantitatively similar to that reported by others [4]. Apparently, the stimulation of actin polymerization during hypotonic incubations or upon addition of glutamine was dependent upon the degree of cell swelling (Fig. 1). The data in Fig. 1 also suggest that the glutamine-effect on the G-/total actin ratio is due to glutamine-induced cell swelling because it can quantitatively be mimicked by equipotent hypotonic cell swelling. On the other hand, the effect of insulin on the G-/total actin ratio was somewhat higher than that observed when equipotent cell swelling was induced by hypotonic exposure. Here apparently also mechanisms distinct from cell swelling come into play, although the insulin effect on the G-/total actin ratio could be explained at least partially on the basis of insulin-induced cell swelling.

### 3.2. *Effects on actin mRNA levels*

As shown in Table II, cell swelling under the influence of insulin, glutamine or hypotonic exposure led to a 2–3-fold increase of actin mRNA levels within 120 min. A comparable extent of actin mRNA increase has been observed in perfused rat liver, when exposed to hypotonic perfusion fluids [12]. It should be noted that the increase in actin mRNA tissue levels was observed regardless of whether reference is made to glyceraldehydphosphate dehydrogenase mRNA, albumin mRNA [12] or to glutamate dehydrogenase mRNA (this paper). On the other hand, the relative actin mRNA level increased only 1.2-fold during hypertonic exposure. Comparing the data given in Tables I and II, it appears that the increase of actin mRNA levels following glutamine and hypotonicity-induced swelling is inversely related to the accompanying decrease of the G-/total actin ratio or the cellular content of G-actin. This would be in line with the recently suggested autoregulatory control of actin synthesis in cultured rat hepatocytes [29] by either the concentration of G-actin [30] or the ratio between monomeric and filamentous actin [29]. Such an autoregulatory control was deduced from the reciprocal behaviour of actin mRNA levels and the G-/F-actin ratio under the influence of clostridium botulinum C2 toxin or the mycotoxin phalloidin [29] and our data would be in line with this suggestion.

Glucagon is known to shrink hepatocytes [27,28] and

Table II

Effects of anisotonicity, glutamine, insulin and glucagon on actin mRNA levels and cellular G-actin content.

For details see Materials and Methods. Data were obtained after 120 min exposure to anisotonicity, glutamine, insulin or glucagon. $\beta$-Actin mRNA levels were normalized for glutamate dehydrogenase mRNA levels.

| | Relative $\beta$actin mRNA level | G-actin content | total actin content |
|---|---|---|---|
| | | $\mu$g/mg cell protein | |
| Control | 1 | 21.2±0.7 (22) | 84.8±1.6 (22) |
| Hypotonic (190 mosmol/l) | 2.4 | 18.4±0.7 (7)* | 86.8±2.4 (7) |
| Glutamine (10 mmol/l) | 2.0 | 17.9±0.6 (5)* | 82.9±2.7 (5) |
| Insulin (100 nmol/l) | 3.6 | 18.1±0.4 (11)* | 84.2±1.9 (11) |
| Hypertonic (430 mosmol/l) | 1.2 | 19.7±1.8 (4) | 82.8±3.3 (4) |
| Glucagon (100 nmol/l) | 2.3 | 20.4±2.4 (3) | 87.9±3.6 (3) |

* denotes statistically significant differences in G-actin content compared to normotonic control incubations ($P < 0.05$). No statistically significant difference was observed for total actin contents.

had, like hypertonic cell shrinkage, no significant effect on the G-/total actin ratio. This is at variance to the observations with glucagon by others [4], however, the actin mRNA level significantly increased under the influence of glucagon (Table II). Thus, apparently the glucagon effects on the microfilament system involve other mechanisms than alterations of the G-actin content.

### 3.3. *Functional significance*

The functional significance of the decrease of G-/total actin ratio following cell swelling, apart from a possible increase of actin mRNA levels, cannot be judged from our present study. However, it is well conceivable that swelling-induced changes in actin polymerization could participate in the modulation of cell function by cell volume. For example, microfilaments are involved in bile formation (for review see [31]) and liver cell volume was recently recognized as a major determinant of transcellular taurocholate transport in liver [32,33]. Further, there is some evidence for a role of microfilaments in regulating other cellular functions, which are also affected by cell volume changes [1,22], such as control of protein synthesis [7], alterations of glycolytic enzyme activities [3] and the regulation of growth-related phenomena [6]. Indeed, microfilament alterations have been recently observed in *ras*-oncogene transformed buffalo rat liver cells [34], while ras oncogene expression in NIH 3T3 fibroblasts is accompanied by a marked increase of cell volume [35].

Taken together our results demonstrate rapid effects of cell swelling on microfilaments. The known insulin effects on microfilaments can at least in part be explained by insulin-induced cell swelling.

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